

# Ultrastructure of *Azotobacter vinelandii*

G. R. VELA, G. D. CAGLE, AND P. R. HOLMGREN

Department of Biological Sciences, North Texas State University, Denton, Texas 76203

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Vegetative cells and cysts of *Azotobacter vinelandii* 12837 were prepared for electron microscopy by several methods assumed to preserve structural details destroyed by techniques previously reported in the literature. Examination of large numbers of cells and cysts by these methods revealed four structural details not reported previously: intine fibrils, intine vesicles, intine membrane, and microtubules. The intine fibrils form a network in the gel-like homogeneous matrix of the CC2 layer. Intine vesicles which seem to originate in the cell wall complex of the central body are seen in the intine and exine of cysts. Analogous structures are found on vegetative cells. The intine is divided into two chemically distinct areas by the two-layered intine membrane. Microtubules, previously reported only in vegetative cells, were found in cysts.

Electron microscopy of thin sections of cells has been used to study the subcellular structure of many bacteria including members of the genus *Azotobacter*. Bisset and Hale (1) published the first electron micrographs of *A. chroococcum* cells. These were gold-shadowed preparations of "gonidia." In 1961 (11), thin sections of the cell and cyst of *A. vinelandii* 12837 were prepared from material imbedded in epoxy resin after  $\text{KMnO}_4$  fixation. These preparations showed that vegetative cells have a granular vacuolated cytoplasm and that cysts contain a central body with nuclear region, lipid inclusions, an intine, and an outer exine. A subsequent report by the same group (15) using essentially the same methods revealed the presence of cytoplasmic membrane, cell wall, nuclear material, and peripheral bodies in vegetative cells and cysts of *A. vinelandii* 12837. These findings were confirmed by Tchan, Birch-Andersen, and Jensen (12), who used cells fixed with  $\text{OsO}_4$  or  $\text{KMnO}_4$  and imbedded in Araldite. They also described the triple-layered lamellar architecture of the cyst exine. Pope and Jurtshuk (7) reported the presence of microtubules in *A. vinelandii* strain O treated with potassium phosphotungstate and glutaraldehyde. The function of these microtubules has not been demonstrated; they were, however, associated only with metabolically active vegetative cells. Koo, Lin, and Sadoff (4, 5) employed carbon replicas to show that the surface structure of vegetative cells walls is smooth and regular, whereas that of the cyst coat is wrinkled and irregularly folded. Lin and Sadoff showed (6) that the exine is a complex multilayered lamellar structure which can be readily separated from the gel-like viscous intine.

This morphology appears to be drastically altered during endogenous metabolism (16).

This report shows additional structural details in *A. vinelandii* 12837 obtained by methods not previously used in the study of *Azotobacter*.

## MATERIALS AND METHODS

**Cells.** *A. vinelandii* 12837 was grown on Burks medium (14) with 1% glucose as carbon source and 2% agar as solidifying agent; glucose was replaced by 0.3% *n*-butanol to induce encystment.

**Electron microscopy.** All vegetative cells were obtained from the Burks glucose medium after 18 to 24 hr of incubation at 30 C, and all cysts from the *n*-butanol medium after 4 to 5 days of incubation at the same temperature. To rule out artifacts, cells from the same culture were treated by the different procedures described below and compared to results obtained with a more conventional method (13). The various methods of pretreatment, fixation, and staining used are described below; ultimately, however, all cells were dehydrated by passage through 30, 50, 75, 95, and 100% ethanol solutions and imbedded in Epon 812 by using graded amounts of propylene oxide as the carrier. Polymerization was accomplished at 60 C in 24 to 48 hr in a drying oven. Thin sections were obtained with the Porter-Blum ultramicrotome equipped with diamond knife. All sections were poststained with uranyl acetate for 45 min and then by lead tartrate for 30 min and examined with an RCA-EMU-3G microscope at magnifications of  $\times 11,000$  diameters.

**Method I.** Cells were removed from the growth medium, washed three times in water, fixed for 1 hr in 3% glutaraldehyde buffered with 0.1 M cacodylate buffer, transferred to Bouin's fluid for 20 min, washed several times with buffer, placed in 1%  $\text{OsO}_4$  in cacodylate buffer for 1 hr at 4 C, and finally washed and dehydrated.

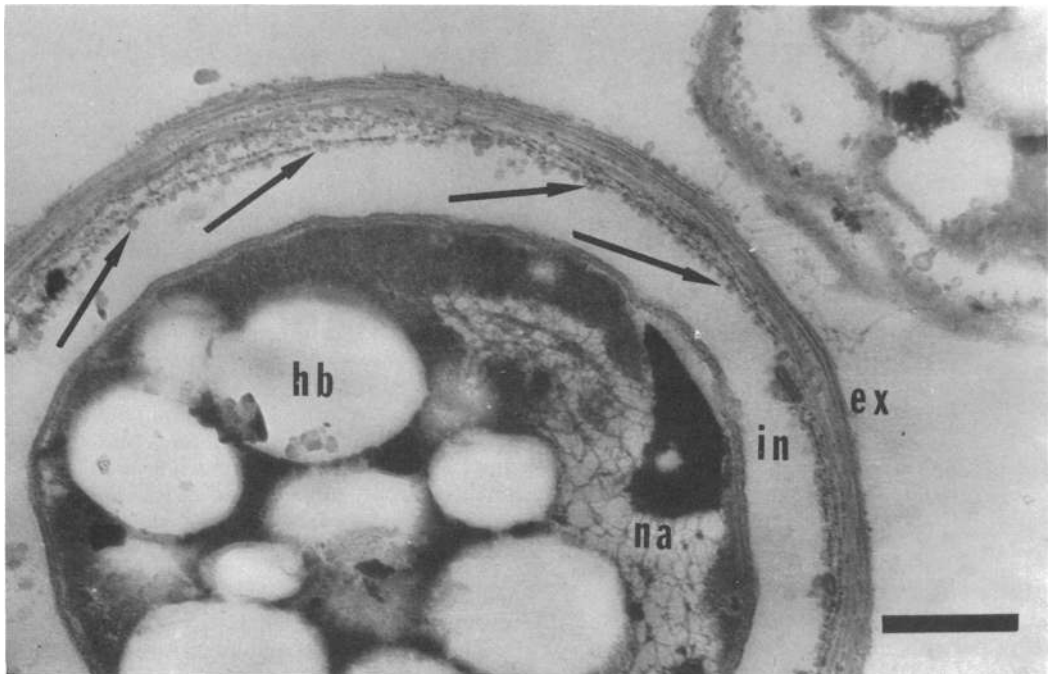


FIG. 1. Intine vesicles in cysts of *Azotobacter vinelandii* treated by method III. Cysts show a clear empty intine space (in) plainly separated from the lamellar exine (ex). Arrows point to the intine vesicles. A reticular structure designated nuclear area (na) occupies a large part of the cell and the large transparent areas are sites where poly- $\beta$ -hydroxybutyrate (hb) was located in the intact cysts. The bar is 0.5  $\mu$ m.

**Method II.** Washed cysts were suspended for 1 hr in 3% glutaraldehyde in a buffer solution containing 0.1 M cacodylate and 1% (w/v)  $K_2Cr_2O_7$  adjusted to pH 7.4 with NaOH (2). They were transferred to a solution of 1%  $OsO_4$  in 0.1 M cacodylate buffer for 1 hr at 4 C, washed, and dehydrated.

**Method III.** Cells or cysts were removed from the growth medium, washed, and lyophilized. They were resuspended in 3% glutaraldehyde buffered with 0.1 M cacodylate buffer, fixed for 1 hr, washed, placed in 1%  $OsO_4$  in cacodylate buffer for another hour at 4 C, and finally washed and dehydrated.

**Method IV.** Lyophilized cells or cysts were treated as described for method I.

## RESULTS

Cysts prepared by method III reveal a clear intine, separate and distinct from the lamellar exine. The exine lamellae appear more organized and compacted at the periphery of the cell than they do adjacent to the intine (Fig. 1). An anatomical detail associated with these structures is seen routinely in cysts and vegetative cells prepared by this method and also by methods I and IV. These structural elements are particulate spherical bodies (intine vesicles) generally seen at the juncture of the intine and exine and on the surface of the central body of cysts (Fig. 1, Fig. 2).

They are also evident in vegetative cells (Fig. 3), in which they appear on the cell wall surface and free in the medium surrounding the cell. They seem to be extruded from the cell membrane-cell wall complex of cells (Fig. 3) and cysts (Fig. 4) outward into the medium. Electron micrographs of cysts treated by method IV (Fig. 5) show that the intine vesicles are circumscribed by a double-layered membrane.

A second structural detail of the intine is shown in Fig. 2. A network of electron-dense fibrils (intine fibrils) imbedded in a less electron-dense matrix is seen only in cysts pretreated with glutaraldehyde and Bouin's fluid. These fibrils have not been specifically shown before, but their existence has been inferred from many electron micrographs (12, 13, 15). Examination of many cysts prepared by method IV, identical to method I except that the cysts are lyophilized, failed to show the intine fibrils. We infer from this observation that intine fibrils form a fragile structural component of the intine which is destroyed by lyophilization.

Method II, considered a carbohydrate stabilizing procedure, was used to study the difference between the CC1 and CC2 layers first described

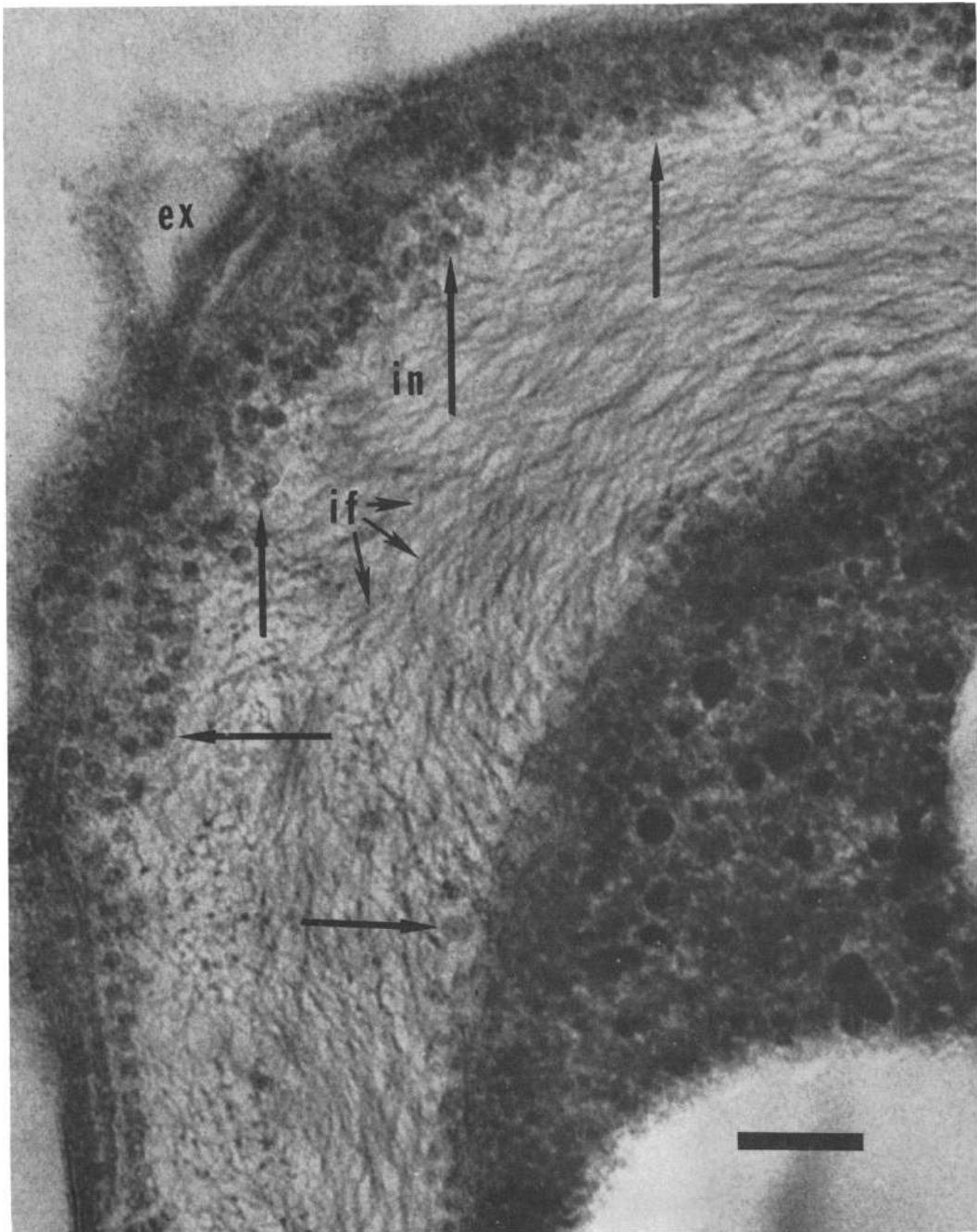
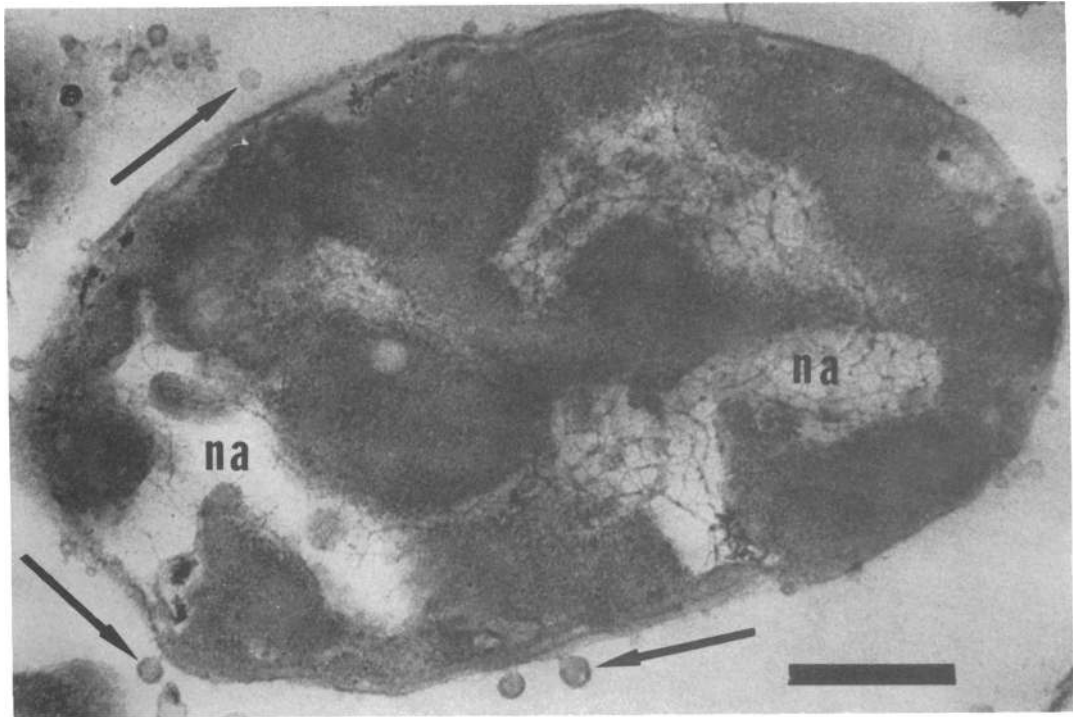


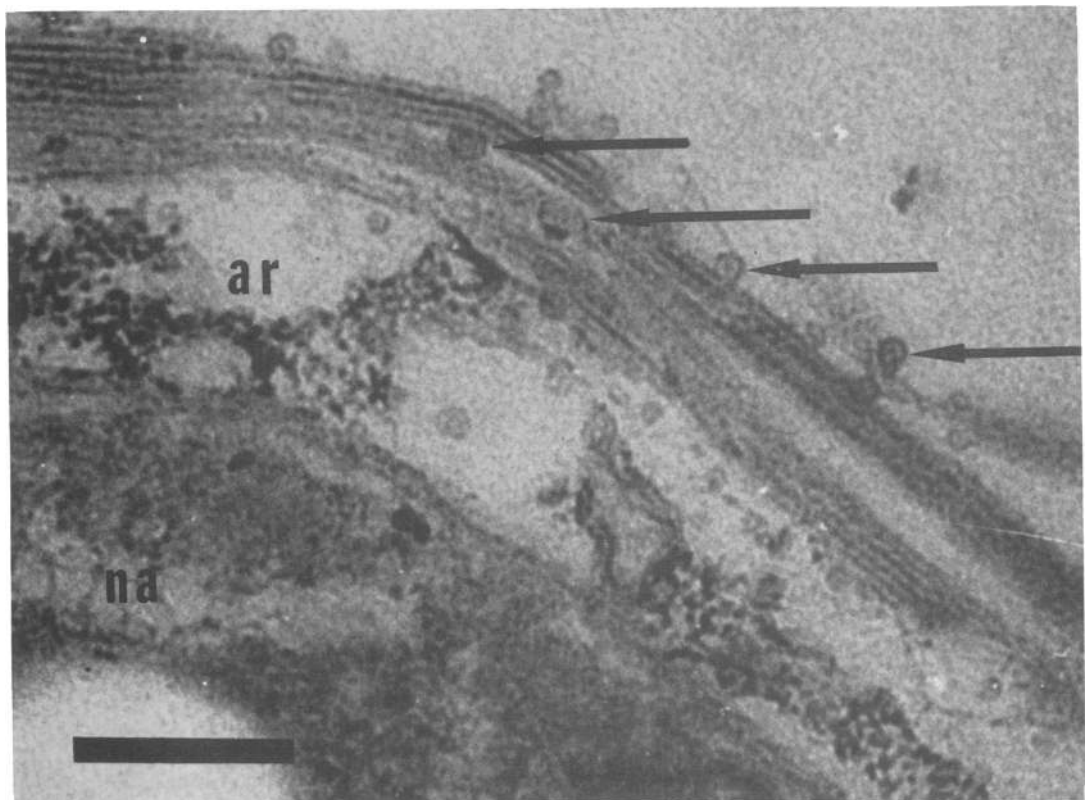
FIG. 2. Cyst treated by method I showing the exine (ex), intine (in), and intine fibrils (if). The long arrows point to intine vesicles. The bar is 0.1  $\mu\text{m}$ .

by Tchan et al. (12). It was found that the two layers are separated by a continuous membrane (Fig. 6) and that the chemical nature of the CC2

layer is such that cysts stained by this method do not show structural elements like those seen in the CC1 layer. The membrane appears to be a



**FIG. 3.** Vegetative cell of *Azotobacter vinelandii* treated by method III. Arrows point to structures very similar to intine vesicles. In vegetative cells, they appear at cell wall and free in the medium. The nuclear area (na) appears identical to that of the cysts and seems to extend the length of the cell. The bar is 0.5  $\mu\text{m}$ .



**FIG. 4.** Cyst treated by method IV shows the intine vesicles (arrows) associated with the outer layers of the exine. The nuclear area is designated na. Dark gray material (ar) in the intine is assumed to be an artifact since it lacked consistency in different preparations. The bar is 0.25  $\mu\text{m}$ .

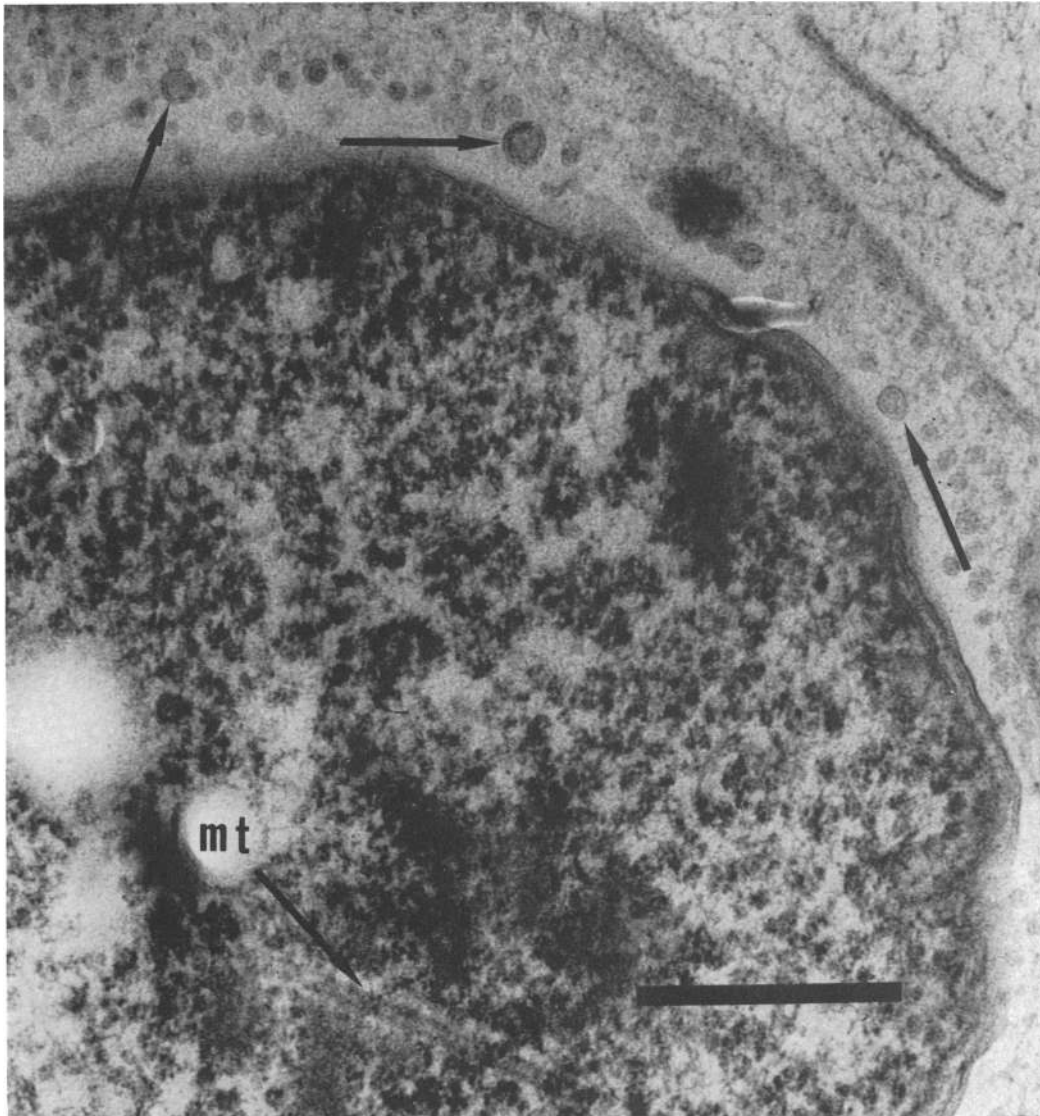


FIG. 5. Cyst treated by method IV. Arrows point to spherical intine vesicles with double-layered surrounding membrane. In lower center is a cyst microtubule (mt). The marker is  $0.25 \mu\text{m}$ .

double-layered structure which effectively separates the intine into two chemically distinct entities, the CC1 and CC2 layers. This membrane could not be observed by the other methods of treatment.

Microtubules, structural elements previously reported only in vegetative cells (7, 9), were observed in cysts treated by method IV (Fig. 5). Only a few normal cysts had microtubules; in a related experiment, however, most cysts of *A. vinelandii* grown in the presence of low concentrations of penicillin contained such micro-

tubules. We infer from this comparison that microtubules can be observed by this method but that normal cysts do not have large numbers of these subcellular elements.

#### DISCUSSION

The use of various treatments to stabilize specific constituents for examination revealed structural details of *A. vinelandii* 12837 not seen in cells prepared by other methods. We chose treatment with glutaraldehyde, assuming that it would preserve structural cell proteins since it has

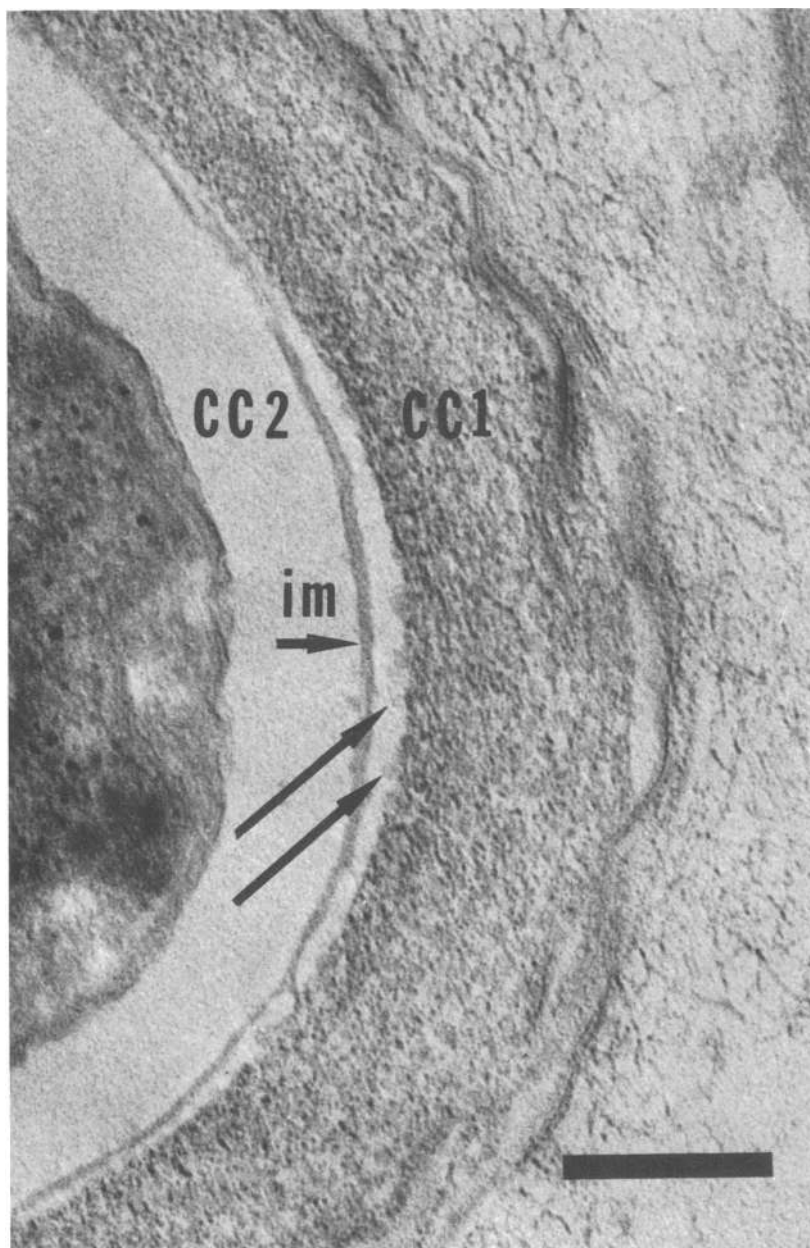


FIG. 6. Cysts stained by method II show the presence of an intine membrane (im) which appears to separate the CC1 and CC2 areas of the intine. Intine vesicles are evident (long arrows) in the CC1 layer but are not seen in the CC2 layer of cysts stained by this procedure. The marker is 0.25  $\mu$ m.

been shown that tissue enzymes continue to function in its presence (9), and lyophilization, because viable cells probably retain sufficient structural integrity to maintain physiological competence. The premise for this work was the fact that  $\text{OsO}_4$  and  $\text{KMnO}_4$  are strong oxidizing agents that undoubtedly alter cell constituents.

It was assumed that glutaraldehyde and Bouin's fluid denature cell proteins and make them chemically more stable, thus, subsequent treatment with  $\text{OsO}_4$  and  $\text{KMnO}_4$  would not bring about extensive destructive oxidation (3, 10).

By using the four methods devised on these assumptions, four structural entities of azoto-

bacter (i) intine fibrils, (ii) intine vesicles, (iii) intine membrane, and (iv) cyst microtubules were discovered.

Intine vesicles (8) appear to be produced by the cell membrane-cell wall complex of cysts and vegetative cells. In cysts, the free vesicles appear to migrate toward the exine and eventually to the cyst exterior. This phenomenon gives the impression that neither intine nor exine are rigid impermeable structures. Spherical structures identical to intine vesicles are also found on vegetative cells (Fig. 3). We infer from this observation that the vesicles are not specific cyst organelles and that the *Azotobacter* cyst is essentially a resting vegetative cell not analogous to bacterial spores. This assumption is further supported by the finding of microtubules in cysts.

The complexity of the cyst coat is indicated by the presence of intine fibrils and the intine membrane. The fibrils are not seen in lyophilized cysts and are presumably destroyed by this manipulation. They appear to be homogeneous with regard to electron density regardless of their position in the intine, whereas the CC1 layer is obviously chemically different from the CC2 layer. Numerous examinations of cysts prepared by method II indicate the presence of a membranous structure separating the CC1 from the CC2 areas of intine. This membrane is double layered and is found in all cysts treated with the chrome-osmium preparation.

The existence of intine vesicles, intine fibrils, and intine membrane in cysts of *A. vinelandii* negates the concept of the cyst coat as simply the polymerized capsule of the vegetative cell. It must be replaced by a concept which is more in keeping with the observations reported by Lin and Sadoff (6), by Pope and Wyss (8), and by us in this work. It is reasonable to assume that the *Azotobacter* cyst is a complex metabolically active entity and that the cyst coat is a complex functional part of the encysted cell.

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